1652 AF



PATENT
2173-0105P

IN THE U.S. PATENT AND TRADEMARK OFFICE

| | | | |
|---|---|---|---|
| In re application of | | Before the Board of Appeals | |
| Yuji HATADA et al. | | Appeal No.: | |
| Appl. No.: | 08/952,741 | Group: | 1652 |
| Filed: | November 25, 1997 | Examiner: | SLOBODYANSKY, E. |
| Conf.: | 3031 | | |
| For: | GENE ENCODING ALKALINE LIQUEFYING ALPHA-AMYLASE | | |

#24
BJG
11/16/01

## APPEAL BRIEF TRANSMITTAL FORM

Assistant Commissioner for Patents
Washington, D.C. 20231:

October 31, 2001

Sir:

Transmitted herewith is an Appeal Brief (in triplicate) on behalf of the Appellants in connection with the above-identified application.

☐ The enclosed document is being transmitted via the Certificate of Mailing provisions of 37 C.F.R. 1.8.

A Notice of Appeal was filed on _____.

☐ Applicant claims small entity status in accordance with 37 C.F.R. § 1.27

The fee has been calculated as shown below:

☒ Extension of time fee pursuant to 37 C.F.R. §§ 1.17 and 1.136(a) - $920.00 three (3) months extension.

☒ Fee for filing an Appeal Brief - $320.00 (large entity).

☒ Checks in the amount of $920.00 and $320.00 totaling $1,240.00 are attached.

☐ Please charge Deposit Account No. 02-2448 in the amount of $0.00. A triplicate copy of this sheet is attached.

11/01/2001 SDIRETA1 00000022 08952741
01 FC:117 920.00 OP

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By _____
John W. Bailey, #32,881

JWB/TBS/crt
2173-0106P

P.O. Box 747
Falls Church, VA 22040-0747
(703) 205-8000

(Rev. 09/27/01)

1483

PATENT
2173-0106P

#25
AQ
11/6/01



IN THE U.S. PATENT AND TRADEMARK OFFICE

| | | | |
|---|---|---|---|
| Applicant: | Yuji HATADA et al. | Conf.: | 3031 |
| Appl. No.: | 08/952,741 | Group: | 1652 |
| Filed: | November 25, 1997 | Examiner: | Slobodyansky, E. |
| For: | GENE ENCODING ALKALINE LIQUEFYING ALPHA-AMYLASE | | |

APPEAL BRIEF

Assistant Commissioner for Patents
Washington, DC 20231

October 31, 2001

Sir:

In response to the Examiner's Office Actions dated June 7, 2001, December 4, 2000, and May 24, 2000, the following Appeal Brief is respectfully submitted in connection with the above-identified application.

**TABLE OF CONTENTS**

I. Real Party in Interest

The real party in interest of the present invention is Kao Corporation of Tokyo, Japan, the assignee of the entire right and interest of the instant application. The assignment of said right and interest was recorded on November 25, 1997 at Reel 9071, Frame 0888.

II. Related Appeals and Interferences

There are no related appeals or interferences pending for the present application.

III. Status of Claims

Claims 2-7, 12-16, and 20-24 are pending in the present application. Claims 2-7, 14-16, and 20-24 are rejected under 35 USC §112, first paragraph and claims 12 and 13 are allowed. The Examiner has indicated in the Advisory Action of June 7, 2001 that claims 2, 5-7, and 14 would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claims and rewriting claims 2, 5-7, and 14 into independent form. Thus, the rejections concerning claims 3, 4, 15, 16, and 20-24 are appealed.

IV. Status of Amendments

Subsequent to the Examiner's final rejection of claims 2-7, 14-16 and 20-24 on December 4, 2000, an after-final response was filed May 31, 2001. An attempt was made amend claims 2, 3, 20, 22, 23, and 24 in the after final response of May 31, 2001, but the Examiner denied entry of the amendments.[1] A Notice of Appeal was filed with the after-final response on May 31, 2001.

V. Summary of Invention

The present invention provides a DNA fragment encoding alkaline liquefying α-amylase protein, having maximal activity at a pH of 8-9, and such alkaline liquefying α-amylase proteins in which one or more amino acids of a specified amino acid sequence are substituted, added, deleted, or inserted, while retaining its enzymatic activity. (See page 11, lines 19-20 and page 11, line 24 to page 12, line 2). Further, the instant invention provides recombinant DNA containing the DNA fragment encoding alkaline liquefying α-amylase, a transformed microorganism harboring the recombinant DNA, as well as a method for producing alkaline liquefying α-amylase using the transformant, and proteins in which one or more amino acids are substituted, added, deleted, or inserted. (See page 5, lines 8-20, and page 11, lines 7-19). The method of the present invention enables mass production of

alkaline liquefying α-amylases useful as a detergent component. (See page 19, lines 6-11).

VI. Issues to be considered

Issue 1

Does the specification, taken with what was known by one of skill in the art at the time of filing the present application, provide adequate written description for claims 3, 4, 15, 16 and 20?

That is, is there adequate written description for:

(a) the structural language "a DNA encoding an α-amylase having an amino acid sequence of SEQ ID NO: 2 with one or more amino acids substituted, added, deleted or inserted" and

(b) the functional language "without changing enzymological properties", having maximal activity "at a pH optimum of 8-9", and having the ability to hydrolyze "1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6)" without hydrolyzing pullulan?

Issue 2

Does the specification, taken with what was known by one of skill in the art at the time of filing the present application, provide adequate written description of the following functional elements

[1] The Appendix cites the claims as appealed.

recited in claim 21:

activity in a stated pH range;

a stated degree of stability in a stated pH;

activity in a stated temperature range of 20°C to 80°C, with a stated optimum temperature range;

stability in the face of a recited treatment;

a selected molecular weight;

a stated isoelectric point;

stability in the presence of certain cations; and

freedom from inhibition from certain chemicals?

Issue 3

Does the specification, taken with what was known by one of skill in the art at the time of filing the present application, provide adequate written description of claims 22-24, which recite the structural elements provided by SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, and SEQ ID NO: 11 and the functional element of "encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9"?

Issue 4

Does the specification, taken with what was known by one of skill in the art at the time of filing the present application, enable the skilled artisan to make and use "an α-amylase having an amino acid sequence of SEQ ID NO:2 with one or more amino acids

substituted, added, deleted or inserted" and having certain recited functional properties?

Issue 5

Does the specification, taken with what was known by one of skill in the art at the time of filing the present application, enable the skilled artisan to make and use an isolated DNA comprising structural elements provided by SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, and SEQ ID NO: 11 and "encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9"?

VII. Grouping of Claims

Appellants respectfully request that the claims be grouped as follows.

Group I - claims 3, 4, 15, 16, and 20

Group II - claim 21

Group III - claims 22-24

Each group of claims raises different issues for consideration by the honorable Board of Patent Appeals and Interferences as follows:

Group I -Issues 1 and 4

Group II - Issues 1, 2, and 4

Group III - Issues 3 and 5

VIII. Arguments

Issue 1

The Specification provides adequate written description of

(a) the structural language "a DNA encoding an α-amylase having an amino acid sequence of SEQ ID NO: 2 with one or more amino acids substituted, added, deleted or inserted" and

(b) the functional language "without changing enzymological properties", having maximal activity "at a pH optimum of 8-9", and having the ability to hydrolyze "1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6)" without hydrolyzing pullulan as well as other functional elements for claims dependent from claim 3.

Applicants assert that claims 3, 4, 15, 16, and 20 are not properly rejected under 35 USC §112, first paragraph for lacking description of a DNA encoding an α-amylase having an amino acid sequence of SEQ ID NO: 2 with one or more amino acids substituted, added, deleted or inserted and further having certain functional properties.

Independent claim 3 reads as follows:

*3. A DNA molecule encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9 and*

> *possessing an amino acid sequence which has been obtained by modifying an amino acid sequence described in SEQ ID NO:2 in a manner* ***in which one or more amino acids are substituted, deleted, or inserted*** *without changing enzymological properties of said amino acid sequence described in SEQ ID NO:2 and hydrolyzes 1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6) and does not hydrolyze pullulan.*

Issue 1 relates to the words "in which one or more amino acids are substituted, deleted, or inserted". The Examiner stated in the Office Action of December 4, 2000:

> *Claim 3 encompasses a great number of α-amylases having unknown structures and possessing the requisite properties. The Examiner is unable to locate adequate support in the specification for such α-amylases. Thus there is no indication that α-amylases having amino acid sequences other than SEQ ID NO: 2 and having the requisite properties were within the scope of the invention as conceived by Applicants at the time the application was filed.*

As an initial matter, Applicants wish to point out that the literal language "in which one or more amino acids are substituted, deleted, or inserted" occurs on page 12, lines 1-3. This indicates that Applicants considered variants of SEQ ID NO: 2 in which the sequence is modified by having one or more amino acids substituted, deleted, or inserted as within the scope of the invention at the time the application was filed.

At issue is whether Applicants had possession of the full-scope of the claimed invention at the time of filing the application. Applicants assert that they did have possession of

the full scope of the claimed invention (for the claims at issue) for the following reasons.

The "Revised Interim Written Description Guidelines Training Materials" (http://www.uspto.gov/web/offices/pac/writtendesc.pdf visited on September 5, 2001) issued by the U.S. Patent and Trademark Office partly in response to the case *University of California v. Eli Lilly*, 43 USPQ2d 1398 (Fed. Cir. 1997) provides examples of what constitutes adequate written description of a cloned DNA invention. On page 53 of the "Revised Interim Written Description Guidelines Training Materials" (hereinafter, written description training materials) appears an example of a product described by function that is analyzed and found to have adequate written description. The claim recited in this example says

> *A protein having SEQ ID NO: 3 and variants thereof that are at least 95% identical to SEQ ID NO: 3 and catalyze [SIC] the reaction of A→B.*

Similar to this claim, the instant claim 3 recites a sequence (i.e. SEQ ID NO:2) and variants thereof (*i.e.* in which one or more amino acids are substituted, deleted, or inserted without changing enzymological properties of said amino acid sequence). Further, the instant claim 3 has language equivalent to the phrase, "catalyze the reaction of A→B". That is, claim 3 recites

> *and hydrolyzes 1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6).*

The Example in the written description training materials indicates that any enzyme in the genus "catalyzes the reaction of A→B". It does not say how well or the rate at which it must catalyze the reaction from A→B. Thus, the Example provides a less stringent limitation than that enumerated in the instant claim 3, which recites a defined level of activity, i.e., "*without changing enzymological properties of said amino acid sequence described in SEQ ID NO:2*". Therefore, claim 3 is in this sense narrower than the "safe harbor" example in the written description training materials.

The analysis in the Written Description Training materials further indicates that only one species was presented in the disclosure. This is similar to the instant invention wherein Applicants have provided one actual species and one hypothetical species. (See page 12, lines 3-6).

The example provided in the written description training materials also has a limitation in it that says the enzyme variants must have 95% sequence identity to the enzyme having the sequence given by SEQ ID NO: 3. The instant claim 3 does not have a limitation that limits the variants to 95% identity in sequence. However, there are three other elements in claim 3 that definitively describe the genus. The first element is that the generated mutant enzyme must have optimal activity at pH 8-9, the second element is that the enzymological properties are the same

as those of an enzyme of SEQ ID NO: 2, and the third element is that the mutant enzyme does not cleave pullulan.

When this is combined with what is known in the art about amylases, Applicants have provided a well-defined genus. The prior art teaches that amylases are known to have four regions, designated regions I-IV, which are highly conserved (see page 13, last line to page 14, line 3).[2] One of skill in the art would recognize that these are regions wherein, if any changes were made at all, one would at most make conservative amino acid substitutions. One or more amino acids would not be inserted or deleted into these regions. Applicants contend that this knowledge to the art, taken with the functional limitations of the claims, is at least equivalent to 95% sequence homology of the example from the written description training materials.

Furthermore, in the example from the training materials, there is no indication if any other enzymes similar to the enzyme from liver being claimed are known. If similar enzymes are not known, then one of skill in the art would not know where to make amino acid substitutions, deletions, or additions and still retain the activity of any mutant enzyme. This is opposite of the case of amylases. Because many amylases are known in the art, one of skill in the art has a starting point from which to direct amino acid substitutions,

deletions, or additions. Further still, claim 3 includes functional limitations as noted above.

When these functional limitations are combined with the structural limitation of variation of SEQ ID NO: 2, a genus is definitively described.

The example presented in the written description training materials also indicates that an assay is known that will allow one to identify the enzymes that have the stated activity. Likewise, the instant specification describes an assay that would allow one to identify enzymes that have the activity recited in the instant claimed invention. See page 17, lines 14-22 in the specification.

Every element that is present in the claim in the Example presented in the written description training materials has a comparable element in the instant claim. The underlying support in the disclosure for the claimed invention in the instant case is equal to or exceeds that support in the written description that is enumerated in the Example. Because the written description training materials expressly indicates that the example has adequate written description support, Applicants assert the present claim 3 is supported by adequate written description.

---

[2] It is noted that Applicants in the instant invention used the sequence of conserved region II to design and isolate the gene that encodes the instant alkaline α-amylase. Also see Attachment I, Nakajima et al., Comparison of amino acid sequences of eleven different α-amylases, Appl. Microbiol. Biotechnol. 23, 355-360, (1986).

Further, the Court of Appeals for the Federal Circuit, in *University of California v. Eli Lilly*, 43 USPQ2d 1398, 1404 (Fed. Cir. 1997) citing *Fiers v. Revel*, 25 USPQ2d 1601 (Fed. Cir. 1993) held that adequate written description of a DNA "requires a precise definition, such as structure, formula, chemical name, or physical properties". The Board should consider that the Federal Circuit used alternative language in enumerating those things that constitute adequate written description. Thus, either a precise definition containing structural properties or a precise definition containing physical properties is sufficient to show adequate written description.

Applicants contend that claim 3 and claims dependent from it are drawn to DNA having both defined structural elements and defined physical properties. The structural element of claim 3 derives from the amino acid sequence of SEQ ID NO: 2. Applicants have further provided an additional example of a species that fits into the claimed genus, i.e. SEQ ID NO: 2 with up to the 32 amino acids deleted from the N-terminus. (See page 12, lines 3-6).

Additional structural information can be gleaned from what was known in the art at the time of filing the application. It has long been held that the specification need not describe that which is known by one of skill in the art. See *In re Buchner*, 18 USPQ2d 1331 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.* 231 USPQ2d (Fed. Cir. 1986) and *Lindemann*

*Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 221 USPQ 481 (Fed. Cir. 1984). This knowledge of the skilled artisan supports written description.

Amylases are known to have four regions, designated regions I-IV that are highly conserved (see page 13, last line to page 14, line 3).[3] Thus, one of skill in the art would recognize that these conserved regions are portions of the sequence in which one would make only conserved amino acid substitutions (if any), and would not insert, delete or substitute one or more amino acids.

Claim 3 also lists the following physical properties;

1) an optimum activity at pH 8-9,

2) no change in enzymological properties from the enzyme having a sequence of SEQ ID NO:2 and

3) the ability of this enzyme to hydrolyze 1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6), and

4) the enzyme does not hydrolyze pullulan.

Claims 4, 15, and 16, that are dependent from claim 3, have additional functional elements that further define subgenera.

---

[3] The Board should note that , in making the instant invention, Applicants used a primer designed from conserved region II that encodes the instant alkaline α-amylase. One of skill in the art can design primers based on conserved regions of proteins because these regions remain almost unchanged throughout "experiments of nature" conducted by evolution. Thus, these regions are thought important for enzyme function.

Claim 4 also has a nucleotide sequence that regulates expression of a gene. Claim 15 is composed of a recombinant DNA containing the DNA of claim 3. Claim 16 is composed of a recombinant DNA containing the DNA of claim 4. Claim 20 has the further functional limitation that the encoded protein has an isoelectric point higher than 8.5 when measured by isoelectric focusing electrophoresis.

Thus, according to *Fiers*, whose holding was cited with favor in *Lilly*, Applicants contend that they have met the written description requirement. In accordance with the rule set out in *Fiers*, Applicants have provided precise definitions in claims 3, 4, 15, 16, and 20 i.e. the recitation of physical properties. Combined with the structural language which derives from SEQ ID NO: 2 and mutant proteins which have amino acids substituted, deleted or inserted provides structural language, Applicants have adequately defined the genus.

In *Lilly*, the Federal Circuit found that the University of California lacked written description because they had only disclosed cDNA for rat insulin in their written description, yet they generically claimed a recombinant plasmid containing cDNA for vertebrate insulin in their claims 1 and 2. The Court found that written description was lacking because the recitation of rat insulin was not sufficient to claim the entire genus of vertebrate insulin.

In *Lilly*, because the University of California had neither structural nor functional (physical properties) language in their claims, the Federal Circuit held that one of skill in the art would be unable to visualize or recognize what constituted the claimed genus.

The Federal Circuit held that the University of California could not have possession of a genus that could not be visualized or recognized. Further, the Federal Circuit found that the University of California had failed to adequately define the genes that fall within its definition. Perhaps their question was, "what is the amino acid sequence of 'vertebrate insulin'? The University of California patent described a sequence for rat insulin gene, but no sequence for any generic insulin, nor were any functional limitations described that indicated what was intended by "insulin".

On the other hand, one of skill in the art would be able to envision and recognize what constitutes the genus of claim 3. There is functional language as well as structural language in the claims, not mere naming of an enzyme as was done in *Lilly*. What is meant by "vertebrate insulin" is far less clear than what is meant by

> *"a DNA molecule encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9 and possessing an amino acid sequence which has been obtained by modifying an amino acid sequence described in SEQ ID NO:2 in a manner in which one or more amino acids are substituted,*

*deleted, or inserted without changing enzymological properties of said amino acid sequence described in SEQ ID NO:2 and hydrolyzes 1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6) and does not hydrolyze pullulan".*

In *Lilly*, it is ambiguous whether "insulin" in the University of California's claims constituted a structural limitation on amino acid sequence or a functional limitation on activity. Furthermore, the scope of any possible functional limitation is open, as there is no specific level of activity recited. The language of the claims in *Lilly* did not indicate that the insulin even had to be active. This is not the case with the instant claim 3.

The instant claim 3 recites cDNA that expresses alkaline α-amylase having the enzymological properties of a protein of SEQ ID NO: 2 and that has maximal activity at pH 8-9. The instant invention further describes how one would screen for enzymes that have maximal activities in the pH 8-9 range (see page 17, lines 14-22). This assay further describes quantitatively how one could measure the activity of these enzymes (one unit activity = amount of protein that produced a quantity of reducing sugar equivalent to 1 μmol of glucose). Further, Applicants have provided how one would screen recombinant microorganisms (see page 9, lines 1-11) to identify those expressing an enzyme according to claim 3.

When the structural features provided by SEQ ID NO: 2, the known conserved regions in amylases, the assays used to screen for mutant enzymes, and the limited number of amino acids (only 21 naturally encoded amino acids are known) are combined, one can only conclude that Applicants did have full possession of the full scope of their claims at the time of filing.

In conclusion, claims 3, 4, 15, 16 and 20 include both structural and functional language that definitively describes the genus encompassed by these appealed claims. The language of these claims allows one of skill in the art to readily visualize and recognize the scope of the generic invention. Applicants have further provided methods for assaying how one tests enzymes that are variants of SEQ ID NO: 2 to easily ascertain species that fall into the claimed genus. Finally, Applicants have provided one species that falls into the claimed genus as well as described modification of those species that would fall into the claimed genus. Accordingly, Applicants have shown that they had possession of the full scope of claims 3, 4, 15, 16, and 20 at the time of filing the invention. It is respectfully requested that the honorable Board reverse the Examiner with respect to the written description rejection of these claims.

Issue 2

The specification, taken with what was known by one of skill in the art at the time of filing the present application, provides

adequate written description of the following functional elements recited in claim 21:

(a) acts in a pH range of 5.0 to 11.0, with an optimum pH in the range of 8.0 to 9.0;

(b) is stable in a pH range of 5.0 to 10.5 and retains at least 50% of activity after treatment at 40°C for 30 minutes;

(c) acts in a temperature range of 20°C to 80°C, with an optimum temperature in the range of 45°C to 55°C;

(d) is stable at temperatures of 50°C or lower when treated for 30 minutes in a glycine-salt-sodium hydroxide buffer having pH 8.5;

(e) has a molecular weight of 50,000+5000 when measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis;

(f) has an isoelectric point of approximately 9.2 when measured by isoelectric focusing electrophoresis;

(g) is stable in the presence of $K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Ba^{2+}$, $Fe^{2+}$, $Fe^{3+}$, or $Al^{3+}$; and

(h) is substantially free of inhibition by surfactants selected from the group consisting of sodium linear alkylbenzene sulfonates, sodium alkylsulfonate esters, sodium polyoxyethylene alkylsulfate esters, sodium alkylsulfonates, soaps and polyoxyethylene alkyl ethers.

Applicants assert that the description of the specification establishes that had possession of the full scope of claim 21 at

the time of filing the application. All of the arguments presented above for Issue 1 apply to this Issue 2 as well. Claim 21 further recites structural and functional elements in addition to the structural and functional elements of claim 3. These elements include a pH range where the alkaline liquefying α-amylase has to be active, a temperature range where it is active, an isoelectric point, stability in the presence of metal ions and surfactants, and a molecular weight range. These elements are expressly described in the abstract of the PCT application from which this application claims benefit. In particular, claim 21 recites a molecular weight range from 45,000 to 55,000 as an additional structural element (also disclosed in the abstract of the PCT application). This molecular weight range apprises one immediately of the collection of amino acids that are possible. In particular, the number of amino acids must be between 241 and 964 amino acids.[4] Every possible combination of amino acids that fits in this genus is immediately conceivable in this case. The additional functional limitations in claim 21 even further limit the genus. For example, polylysine of 241 to 964 amino acids would not have the requisite isoelectric point.

When the above-enumerated functional elements are combined with the written description of SEQ ID NO: 2, the conserved

[4] These numbers were obtained by taking 45,000g/mol/186.21g trp/mol trp (tryptophan is the heaviest amino acid) and 55000g/mol/57.05g gly/mol gly (glycine is the lightest amino acid).

regions in amylases, the assays used to screen for mutant enzymes provided by the specification, and the limited number of amino acids (21 naturally encoded amino acids), one can only conclude that the claimed invention is well described by the specification. Further, it is clear that Applicants had full possession of the full scope of claim 21 at the time of filing of the instant application. It is respectfully requested that the honorable Board reverse the Examiner with respect to the written description rejection of claim 21.

Issue 3

Claims 22-24, reciting a DNA molecule encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9, comprising at least one nucleotide sequence selected from a group of recited short sequences are not properly rejected under 35 USC §112, first paragraph for lacking description.

In *Fiers v. Revel*, 25 USPQ2d 1601 (Fed. Cir. 1993) the Federal Circuit held that adequate written description of a DNA "requires a precise definition, such as structure, formula, chemical name, or physical properties". As was previously explained, the Federal Circuit used alternative language in enumerating those things that constitute adequate written description. Thus, either a structural definition or a functional

definition is sufficient to provide adequate written description. The present specification provides both of these in claims 22-24. Thus, claims 22-24 are adequately described in the specification.

Claims 22-24, which are directed to cloned DNA encoding an enzyme, have both structural and functional language. The structural element comes from sequences that comprise a primer or the reverse complement of the complementary strand primer. The alkaline α-amylase activity of the encoded enzyme and its maximal activity at pH 8-9 of the enzyme encoded by the claimed DNA provides functional limitations.

The genus defined by claims 22-24 has a definitive structural element (i.e. short DNA sequences composed of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, or SEQ ID NO: 11). There is no ambiguity in this structural element. No variants in this structural element are claimed. The DNA that encodes the alkaline liquefying α-amylase, must have at least one of these enumerated sequences (or the reverse complement) present in them. The manner in which these short sequences relate to the entire structure of a DNA encoding an α-amylase of the invention is shown by use of primers of the recited sequences to isolate a DNA of the invention. Thus, Applicants have defined a genus that can be immediately visualized or recognized. On this basis alone, Applicants have satisfied the Federal Circuit's requirement that one of skill in the art be able

to visualize or recognize what constituted the claimed genus. See *University of California v. Eli Lilly*, 43 USPQ2d 1398 (Fed. Cir. 1997).

However, claims 22-24 contain additional functional features that further define the invention. The encoded protein must have α-amylase activity and this activity must be maximal at a pH from 8 to 9. What is meant by "alkaline liquefying activity" is defined in the specification at page 3 lines 5 *et seq.* of the specification and an exemplary assay is provided at page 17, lines 14-22. The skilled artisan in enzymology well understands what is meant by a pH optimum of an enzyme activity.

In view of the above, the Board must conclude that the specification adequately describes the subject matter of claims 22-24 and that Applicants were in possession of an invention having the full scope of these claims. It is respectfully requested that the honorable Board reverse the Examiner with respect to this written description rejection.

Issue 4

The specification, taken with what was known by one of skill in the art at the time of filing the present application, enables the skilled artisan to make and use "an α-amylase having an amino acid sequence of SEQ ID NO:2 with one or more amino acids

substituted, added, deleted or inserted" and having certain recited functional properties.

Applicants assert that claims 3, 4, 15, 16, and 20-21 reciting

> *a DNA encoding an α-amylase having an amino acid sequence of SEQ ID NO:2 with one or more amino acids substituted, added, deleted or inserted with maximal activity at a pH optimum of 8-9 and possessing an amino acid sequence which has been obtained by modifying an amino acid sequence described in SEQ ID NO:2 in a manner in which one or more amino acids are substituted, deleted, or inserted without changing enzymological properties of said amino acid sequence described in SEQ ID NO:2 and hydrolyzes 1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6) and does not hydrolyze pullulan*

are improperly rejected under 35 USC §112, first paragraph for not being fully enabled.

Regarding claims 3, 4, 15, 16, and 20-21, the Examiner recites in the Office Action of May 24, 2000:

> *The claims are broader than the enablement provided by the disclosure with regard to the huge number of all possible nucleic acid sequences encoding α-amylase having the specific desired characteristics.*

Applicants submit that the Examiner has failed to meet the burden of presenting a *prima facie* case as to why the claims would not be enabled. See *In re Wright*, 27 USPQ2d 1510 (Fed. Cir. 1993). *Wright*, citing *In re Marzocchi*, 169 USPQ 367, 369 *(CCPA 1971)* states

> *When rejecting a claim under the enablement requirement of section 112, the PTO bears an initial burden of setting forth*

> *a reasonable explanation as to why it believes that the scope of protection provided by that claim is not adequately enabled by the description of the invention provided in the specification of the application; this includes, of course, providing sufficient reasons for doubting any assertions in the specification as to the scope of enablement. If the PTO meets this burden, the burden then shifts to the applicant to provide suitable proofs indicating that the specification is indeed enabling.*

The Examiner has failed to meet this initial burden. Even if the Examiner had met this burden, Applicants have provided an example that works. Absent some evidence from the Examiner that any mutant enzyme would not work, one must assume that the full scope of the claimed invention is enabled by the specification. Consequently, claims 3, 4, 15, 16, and 20-21 are enabled for the full scope of the invention.

The Court of Appeals for the Federal Circuit in *In re Wands*, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988) stated

> *Enablement is not precluded by the necessity for some experimentation such as routine screening. However, experimentation needed to practice the invention must not be undue experimentation. The key word is 'undue', not 'experimentation'.*

Applicants establish below that the amount of experimentation needed to practice the full-scope of the claimed invention is not 'undue'. Therefore, the present claims should be considered enabled by the present specification.

The Federal Circuit, in *Wands*, enumerated factors to be considered to ascertain whether or not claims are enabled. See *In*

*re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988) at page 1404. These factors are:

1) the nature of the invention,
2) the breadth of the claims,
3) the quantity of experimentation needed to make or use the invention based on the disclosure of the invention
4) the amount of direction provided by the inventor,
5) the presence or absence of working examples,
6) the state of the prior art,
7) the relative skill of those in the art, and
8) the level of predictability in the art.

First, the nature of the invention is such that one of ordinary skill in the art would be able to make and use the invention commensurate in scope with the claims. The invention is cloned DNA encoding an α-amylase enzyme. Practice of the invention involves techniques such as the polymerase chain reaction (PCR), site directed mutagenesis, gene-splicing, and other associated techniques to generate mutant enzymes. Recombinant DNA manipulation techniques, such as site-directed mutagenesis and PCR are well known and routine in the art. In fact, kits for performing these techniques are commercially available. These kits allow one to practice the invention easily. Further, the disclosure has provided guidance as to how one would

go about practicing the invention, including how one would screen for microorganisms to identify those harboring DNA of the invention (see page 9, lines 1-11) and how one would test for activity of the encoded protein (see page 17, lines 14-22).

Second, the breadth of claims 3, 4, 15, 16, 20, and 21 is large, encompassing many variations of SEQ ID NO: 2. However, the breadth of these claims is considerably constrained by the functional limitations recited in claim 3 as the particulars of the activity of the encoded enzyme. An important limitation is that the enzymological properties of the enzyme must be the same as those of a protein having the amino acid sequence of SEQ ID NO: 2. One of skill in the art can readily determine, by the assay described at page 17, lines 14-22, whether any variant of SEQ ID NO: 2 is the same as the parental enzyme in this regard and thus the breadth of claim 3 is considerably reduced. The breadth of claim 3 is further reduced by a limitation that the maximum activity be observed at pH 8-9, that certain recited substrates are hydrolyzable by the enzymes produced, and that one substrate is not hydrolyzable.

Third, the state of the prior art also would allow one to make and use the invention commensurate with the claimed invention. α-amylases are well known in the prior art. There are known to be four conserved regions (designated regions I-IV) in α-amylases. One of skill in the art would recognize that these are

not regions where one would be likely to make additions, substitutions (other than perhaps conservative substitutions) and deletions. The inventors of the instant invention have further used one of these conserved regions (region II) to design a primer so that the gene encoding the alkaline liquefying α-amylase could be isolated. Thus, one of skill in the art would recognize that knowledge in the prior art is sufficiently high that the skilled artisan would be able to practice the invention commensurate in scope with the claimed invention.

Fourth, it is generally acknowledged that the level of skill in the biotechnology art is high. The Federal Circuit, in *Wands*, said that the level of skill in the antibody art was high. Likewise, it is likely that one would consider the skill of someone in the recombinant art to also be high, because the arts often overlap. Usually, the practicing artisan possesses a Ph.D. The Patent Office itself calls the recombinant art "a complex technology". Accordingly, the skill of those who practice in this art must necessarily be advanced in order to practice this "complex technology".

Fifth, the next *Wands* factor is predictability in the art. Applicants admit one can not immediately tell from the primary amino acid sequence whether or not a given amino acid sequence for a liquefying α-amylase would be active at the same level as an

enzyme having the amino acid of SEQ ID NO:2. Thus, the predictability of function from primary structure is low.

However, obtaining an active and operable embodiment of the invention can easily be achieved by screening a library of mutants by the method indicated on page 17, lines 14-22 of the instant specification. The holding in *Wands* expressly stated that such screening was not "undue" experimentation. Screening is expected in the molecular biology art. Applicants, in this particular instance, have devised a spectrophotometric means of testing any possible mutant that allows for high throughput screening of mutants (see page 17, lines 14-22). Use of spectrophotometric assays for screening are routine in the art.

Further, recombinant techniques such as site-directed mutagenesis, the polymerase chain reaction and other recombinant techniques, and sequencing are well known and quite predictable. Kits are common in the art that allow one to practice these techniques and obtain consistent and positive results time after time. Further, Applicants have provided disclosure how one would select the mutants (see page 9, lines 1-11). As to the limitation on isoelectric point (pI) in claim 21, a theoretical pI is very simple to calculate from an amino acid sequence. Computer programs were available at the time filing the application that allowed one to calculate pIs from an amino acid sequence. See attachment II Skoog et al., Calculation of the isoelectric points

of polypeptides from the amino acid composition, *Computer Corner*, 5(4), (1986). Thus, even though predictability may be low based on the primary amino acid sequence, the screening techniques that are used to discover active enzyme variants are trivial. Applicants submit that it is highly likely that at least one active variant enzyme would be isolated in any single experiment of this type. Thus, predictability of success in a "mutation-screening" experiment is high.

Sixth, Applicants have provided sufficient direction so that one of skill in the art could practice the invention. In particular, Applicants have provided assays as to how mutant microorganisms can be selected and also how the mutant enzymes can be assayed to ascertain their activity (see page 9, lines 1-11 and see page 17, lines 14-22, respectively). Recombinant techniques, such as site-directed mutagenesis are well known in the art and thus it would have been redundant for Applicants to have detailed these techniques in detail in the written description. See *In re Buchner*, 18 USPQ2d 1331 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.* 231 USPQ2d (Fed. Cir. 1986) and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 221 USPQ 481 (Fed. Cir. 1984).

Further, because the level of skill is high in the recombinant art, and because the amount of guidance or direction needed to enable the invention is inversely related to the amount

of knowledge in the state of the art, a lot of guidance is not necessary (see *In re Fischer*, 166 USPQ 18, 24 (CCPA 1970)). Accordingly, Applicants have provided sufficient guidance so that one of skill in the art would be able to practice the full scope of the claimed invention.

Seventh, Applicants have provided one working example of an enzyme that cleaves certain carbohydrate bonds recited in the claims and operates maximally at pH 8-9. A screening method that is simple to perform is demonstrated. Recombinant techniques to generate variant enzymes starting from the cloned DNA obtained in the working example are well known. One of skill in the art would recognize that changes should not be made in parts of the sequence that are highly conserved (such as regions I-IV). Further, the claims have constraints on them that would direct one to the sequences that fall into the claimed genus, such as the activity being at a maximum from 8-9, the enzyme retaining its enzymatic activity, and the enzyme cleaving 1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6) and does not hydrolyze pullulan. In view of the above, the one working example provided should be sufficient to justify a generic claim encompassing the disclosed sequence and similar analogues.

Eighth, the quantity of experimentation needed to make and use the invention based on the disclosure is not overly large. Following the working examples of the specification, the prototype DNA of SEQ ID NO: 2 can be obtained from a deposited *Bacillus* strain in a few days time. Mutation and screening experiments as expected in the art can typically be performed within an additional week or two. A skilled artisan in molecular biology does not consider this a large amount of experimentation. The predictability of recombinant DNA manipulation and screening techniques used to practice the invention is also high, and importantly such experiments are expected to be performed by the skilled artisan. The guidance provided by the disclosure is sufficient so that one of skill in the art could practice the invention without designing any new assay. Applicants have described how one would select for mutant microorganisms and Applicants have provided how one would assay any mutant enzyme to test for activity, a constraint on the breadth of the claims (see page 9, lines 1-11 and see page 17, lines 14-22, respectively). The prior art provides guidance as to structural portions of the enzyme that are important to conserve. In view of the above, the experimentation that would be necessary to practice the invention commensurate in scope with the claims would not be undue. Thus, the claimed invention is fully enabled throughout the full scope of the claims. It is respectfully requested that

the honorable Board reverse the Examiner with respect to the enablement rejection of claims 3, 4, 15, 16 and 20-21.

Issue 5

Claims 22-24 claiming DNA molecules encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9, comprising at least one nucleotide sequence selected from the group consisting of the primers disclosed in the invention or the reverse complement of primers disclosed from the complementary strand are improperly rejected under 35 USC §112, first paragraph for lack of enablement.

The Examiner in the Office Action of December 4, 2000 recites

> . . . *the specification . . . does not reasonably provide enablement for a DNA comprising a fragment of about 20 base pairs*

Applicants submit that the Examiner has failed to meet the burden of presenting a *prima facie* case as to why the claims would not be enabled. See *In re Wright*, 27 USPQ2d 1510 (Fed. Cir. 1993). *Wright*, citing *In re Marzocchi*, 169 USPQ 367, 369 *(CCPA 1971)* states

> *When rejecting a claim under the enablement requirement of section 112, the PTO bears an initial burden of setting forth a reasonable explanation as to why it believes that the scope of protection provided by that claim is not adequately enabled by the description of the invention provided in the specification of the application; this includes, of course, providing sufficient reasons for doubting any assertions in the specification as to the scope of enablement. If the PTO*

*meets this burden, the burden then shifts to the applicant to provide suitable proofs indicating that the specification is indeed enabling.*

The Examiner has failed to meet this initial burden. Even if the Examiner had met this burden, Applicants have provided an example that works. Absent some evidence from the Examiner that any mutant enzyme would not work, one must assume that the full scope of the claimed invention is enabled by the specification. Consequently, claims 22-24 are enabled for the full scope of the invention.

Furthermore, consideration of the *Wands* factors will show that undue experimentation is not required to practice the invention set forth in claims 22-24. The nature of the invention, the level of skill in the art, the predictability of the art, the state of the prior art and the amount of experimentation to practice the invention are all the same as for claims 3, 4, 15, 16, and 20-21. Thus the arguments on these points presented as to Issue 4 apply here as well.

The breadth of claims 22-24 differs from that of the other claims on appeal. Claims 22-24 recite specific structural features, i.e. inclusion of certain short nucleotide sequences that are not recited in the other claims. The Board is reminded that the DNA claimed in claims 22-24 comprises these sequences. One of skill in the art recognizes that it is not likely that a

DNA including only these sequences would encode an enzyme possessing α-amylase activity having an optimum level at pH 8-9. Rather, many more amino acids would be needed to complete the enzyme structure.

The disclosure of the specification describes this. In particular, the working examples show that the short DNA sequences recited in the claims are used in a PCR reaction upon a template DNA from some bacterium that expresses a relevant enzyme. The product is the DNA of the invention. (See, Examples 4-6).

Bacteria expressing the relevant enzyme can be identified by Southern Blotting with DNA of "Fragment A", described in Figure 1 (See p. 14, line 12 and Example 3). Species of bacteria that are also possible candidates for use in this process are described on page 2 of the specification. As described above, the assay for activity described in the application allows the skilled artisan to easily distinguish a DNA that encodes an enzyme having alpha-amylase activity, with optimum activity at pH 8-9, from embodiments outside the scope of the claims.

In view of the above, the invention of claims 22-24 should be considered well enabled by the instant specification. Accordingly, the rejection of claims 22-24 under 35 U.S.C. § 112, first paragraph, for alleged lack of enablement, should be reversed. Thus, it is respectfully requested that the honorable Board reverse the Examiner with respect to this enablement

rejection.

IX. Conclusion

For the reasons advanced above, it is respectfully submitted that all claims in this application are allowable. Thus, favorable reconsideration and reversal of the Examiner's rejection of claims 3, 4, 15, 16, and 20-24 under 35 U.S.C. § 112, first paragraph, for alleged lack of written description and for alleged lack of enablement, by the Honorable Board of Patent Appeals and Interferences, is respectfully solicited.

The required Appeal Brief fee in the amount of $320.00 and a three-month extension fee in the amount of $920.00 is attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By: _____
John W. Bailey
Reg. No. 32,881

P.O. Box 747
Falls Church, VA 22040-0747
(703) 205-8000

JWB/TBS/crt
0014-0188P

Attachment: APPENDIX
Attachment

X. Appendix (Appealed claims) isolated

3. (Twice Amended) A DNA molecule encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9 and possessing an amino acid sequence which has been obtained by modifying an amino acid sequence described in SEQ ID NO:2 in a manner in which one or more amino acids are substituted, deleted, or inserted without changing enzymological properties of said amino acid sequence described in SEQ ID NO:2 and hydrolyzes 1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6) and does not hydrolyze pullulan.

4. A DNA molecule as defined in any of claims 2 or 3 (5) further comprising a nucleotide sequence for regulating expression of a gene.

15. A recombinant DNA containing the DNA molecule of claim 3.

16. A recombinant DNA containing the DNA molecule of claim 4.

20. (Amended) The DNA molecule of claim 3, wherein said encoded protein has an isoelectric point higher than 8.5 when

measured by isoelectric focusing electrophoresis.

21. The DNA molecule of claim 3, wherein said encoded protein:

acts in a pH range of 5.0 to 11.0, with an optimum pH in the range of 8.0 to 9.0;

is stable in a pH range of 5.0 to 10.5 and retains at least 50% of activity after treatment at 40°C for 30 minutes;

acts in a temperature range of 20°C to 80°C, with an optimum temperature in the range of 45°C to 55°C;

is stable at temperatures of 50°C or lower when treated for 30 minutes in a glycine-salt-sodium hydroxide buffer having pH 8.5;

has a molecular weight of 50,000±5000 when measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis;

has an isoelectric point of approximately 9.2 when measured by isoelectric focusing electrophoresis;

is stable in the presence of $K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Ba^{2+}$, $Fe^{2+}$, $Fe^{3+}$, or $Al^{3+}$; and

is substantially free of inhibition by surfactants selected from the group consisting of sodium linear alkylbenzene sulfonates, sodium alkylsulfonate esters, sodium polyoxyethylene alkylsulfate esters, sodium alkylsulfonates, soaps and

[5] Note that although this claim is dependent from both 2 and 3, only the claim as it pertains to 3 is being appealed.

polyoxyethylene alkyl ethers.

22. (Amended) A DNA molecule encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9, comprising at least one nucleotide sequence selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 7, SEQ ID NO: 3, SEQ ID NO: 6 and SEQ ID NO: 9.

23. (Amended) A DNA molecule encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9 comprising at least one nucleotide sequence that is the reverse complement of a sequence selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 11.

24. (Amended) A DNA molecule encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9 comprising at least one nucleotide sequence selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 7, SEQ ID NO: 3, SEQ ID NO: 6 and SEQ ID NO: 9, and also comprising at least one nucleotide sequence that is the reverse complement of a sequence selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 11.

Appl Microbiol Biotechnol (1986) 23:355–360

(ATTACHMENT I)

Applied and Microbiology Biotechnology



# Comparison of amino acid sequences of eleven different α-amylases

**Ryoichi Nakajima, Tadayuki Imanaka, and Shuichi Aiba**

Department of Fermentation Technology, Faculty of Engineering, Osaka University, Yamada-oka, Suita-shi, Osaka 565, Japan

**Summary.** A comparison was made of the amino acid sequences of 11 different α-amylases. The 6 animal α-amylases tested were found to be highly homologous (about 80 to 90%, depending on the species compared). Amino acid sequence of *Bacillus stearothermophilus* α-amylase was fairly homologous (about 60%) with that of a thermostable α-amylase from *Bacillus amyloliquefaciens*. Homology was least among the thermolabile amylases from *Bacillus subtilis, Aspergillus oryzae,* plants and animals. Nevertheless, four highly homologous regions were found in the amino acid sequences of all the enzymes, despite their widely different origins. It was inferred that these four homologous regions were likely to be the active and/or substrate-binding sites.

## Introduction

α-Amylase (1,4-α-D-glucan glucanohydrolase; EC 3.2.1.1) is a widely distributed secretory enzyme. The enzyme has been investigated extensively from various aspects: its protein structure and function, its mechanism of secretion through cell membrane, and its industrial application. Several workers have already published amino acid sequences of α-amylase, either deduced from the nucleotide sequences of the cloned genes (Hagenbüchle et al. 1980; MacDonald et al. 1980; Rogers and Milliman 1983; Takkinen et al. 1983; Yamazaki et al. 1983; Yang et al. 1983; Nakamura et al. 1984; Nakajima et al. 1985) or by direct determination by amino-acid analysis (Kluh 1981; Toda et al. 1982).

*Offprint requests to:* T. Imanaka

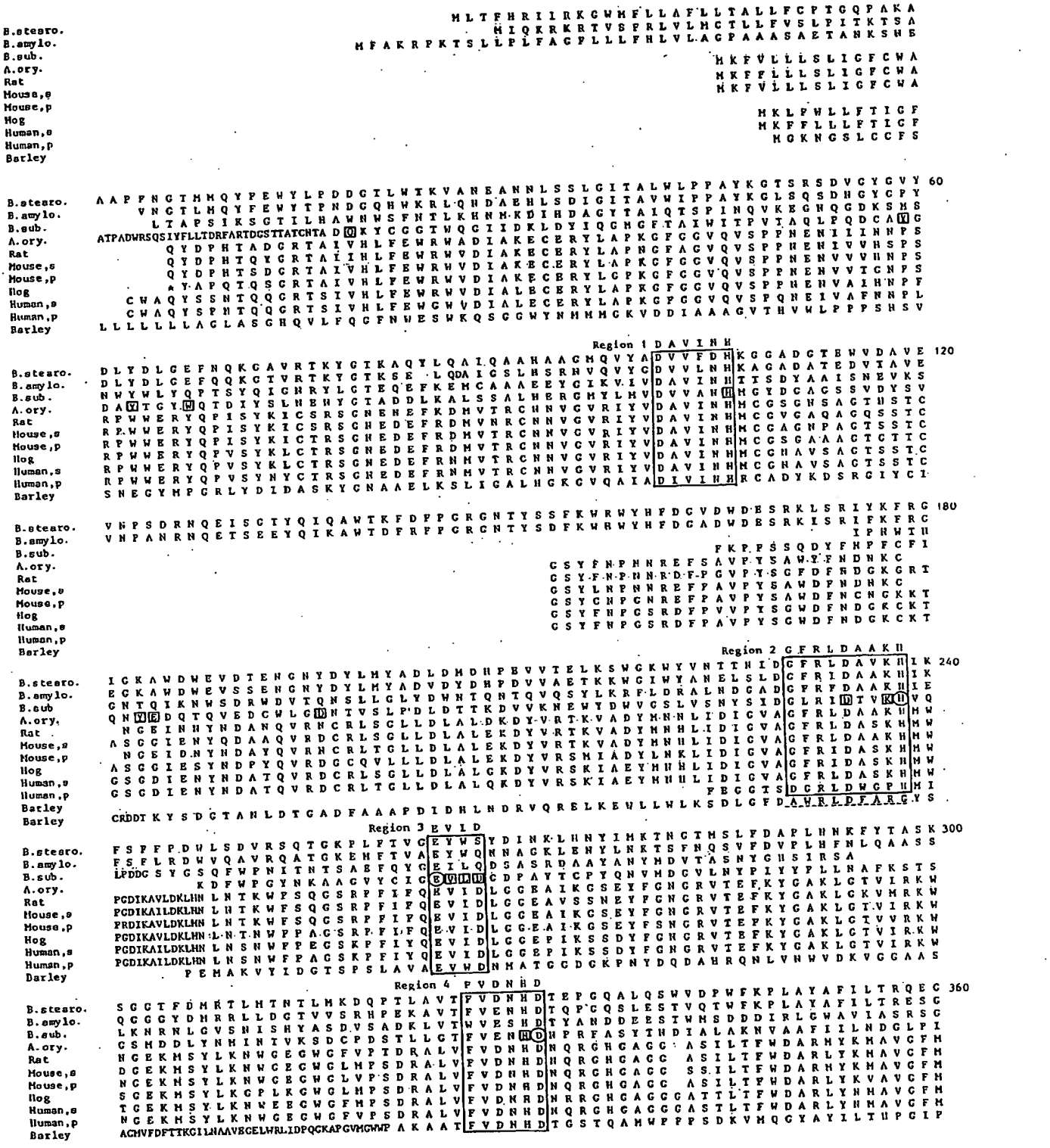
The amino acid sequences and structural properties of a large number of different proteins are to be found in the extensive literature on the subject (Kyte and Doolittle 1982). If this information could be made available, it would in principle be possible to account for the difference in heat resistance shown by the same enzyme from different origins; that is, from organisms that are thermophiles, mesophiles or psychrophiles. Whatever the principle might be, the feature which determines the thermophilic nature of a protein has not yet been made clear. Some reports suggest that thermostability of a protein can be enhanced by a single amino-acid substitution (Yutani et al. 1977; Matsumura et al. 1984).

The purpose of this analysis was to align the known amino acid sequences of α-amylases from different sources in an attempt to detect any regions common to all the enzymes, however, different their origins.

## Procedures

*Alignment of amino acid sequences.* The amino acid sequences of α-amylases shown in Fig. 1 were deduced from a determination of nucleotide sequences from several publications (Hagenbüchle et al. 1980; MacDonald et al. 1980; Rogers and Milliman 1983; Takkinen et al. 1983; Yang et al. 1983; Nakamura et al. 1984; Nakajima et al. 1985). This does not apply to amino acid sequences of hog pancreatic α-amylase and Taka-amylase of *Aspergillus oryzae:* which were determined directly from their cyanogen bromide digests after preparing crystalline samples (Kluh 1981; Toda et al. 1982).

The sources of α-amylase are given on the left of the figure and the sequences are read from left to right. Except for barley, the signal peptide regions are placed in the 1st row of each sequence. Each row for the same enzyme origin is composed, unless otherwise noted, of 60 amino acids from left to right, and the last amino acid is joined sequentially by the first one in the next row.



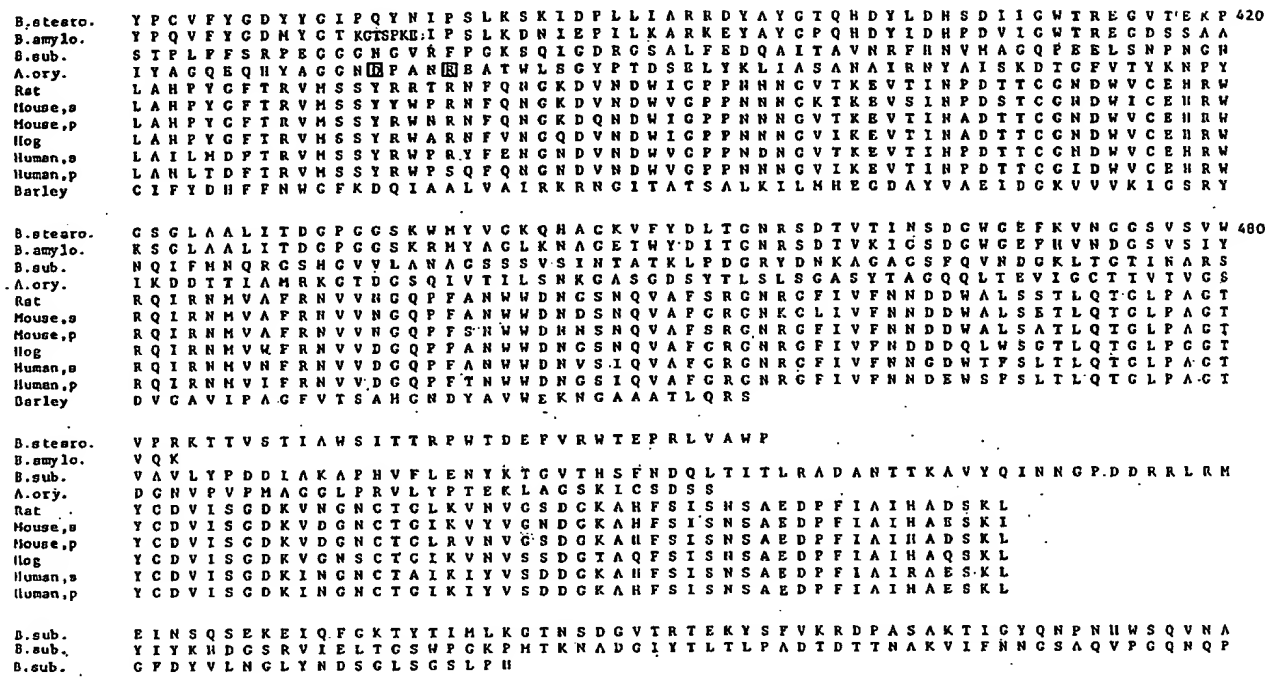


Fig. 1. Comparison of amino acid sequences of various α-amylases. Amino acid residues are shown by single letters as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. Enzyme sources are abbreviated as: B. stearo., *Bacillus stearothermophilus*; B. amylo., *Bacillus amyloliquefaciens*; B. sub., *Bacillus subtilis*; A. ory., *Aspergillus oryzae*; s, saliva; p, pancreas. The first amino acid of extracellular amylase of *B. stearothermophilus*, Ala, is counted as +1. Signal peptides are shown in the first rows. Homologous sequence regions 1, 2, 3 and 4 are surrounded by rectangles. Amino acid sequence described above the rectangular regions was taken as representative of regions 1 to 4, respectively. Active sites and those of substrate binding proposed by Matsuura et al. (1984) for Taka-amylase A from *A. oryzae* are indicated by ○, and □, respectively. Barley amylase has another sequence somewhat similar to region 2 as shown by a broken underline. *, Pyrrolid-2-one-5-carboxylic acid (Kluh 1981)

Some anomalies of alignment are noted in Fig. 1. The interval of arrangement between the neighbouring amino acids is halved and/or expanded, whenever required, to the extent of considerable or total absence of amino acids in some rows of this alignment (see the dented area of the left-hand side of the figure). These anomalies arose either from a trial and error or from a computer-aided search for sequential regions common to all of the enzymes studied (regions 1 to 4, designated by rectangular areas in Fig. 1; for details of the location of these regions, see later). Thus the absence of some amino acids from rows of the same origin does not have any special significance; it has resulted principally from an attempt to facilitate the comparison.

With regard to α-amylase from barley which is composed of 438 amino acid residues, a converse alignment was attempted, after having fixed their regions 1 to 4 (loc. cit.). Accordingly, 11 amino acid residues appearing in the 1st row have nothing to do with the signal sequence in this case. The double rows for barley in the 5th segment from the top likewise are of no special significance: this duplicate row was introduced to extract a segment (with a broken line beneath it) that was homologous with the corresponding regions of other sources (region 2 in Fig. 1). Similarly, the arrangement of triple rows for *B. subtilis* in the last segment of this arrangement was necessary because this α-amylase, composed of 660 amino acid residues, is characterized by a very long sequence in the COOH-terminal region. It is reported that at least 101 amino acid residues of the COOH-terminus could be removed without the loss of the enzyme activity (Yamazaki et al. 1983).

Finally, it should be mentioned that signal sequences of mouse salivary and pancreatic α-amylases shown in Fig. 1 are in the signal sequence for the rat enzyme (MacDonald et al. 1980; MacDonald et al. 1982); Hagenbüchle et al. (1980) have described the signal sequence, comprising the first 12 amino acid residues, for mouse enzyme. By the same token, signal sequences of the human salivary and pancreatic α-amylases might well be altered in the same way; this has not been done, however, and we use the sequence shown in the original paper (Nakamura et al. 1984). However inconsistent this treatment might appear, the arrangement as in Fig. 1 would not have any serious effect on the conclusions to be drawn from this comparison of 11 different α-amylases, or on the identification of homologous regions.

*Analysis of the primary-structure homology and the hydropathic character of α-amylase.* The search for homologous regions in amino acid sequences was aided partly by an NEC PC-8001 computer (Nippon Electric Co., Tokyo, Japan), using a program of the dot matrix (Novotny 1982). The hydropathic character of α-amylase of *B. stearothermophilus* (taken as an example) was also analysed by the computer as described earlier (Matsumura et al. 1984).

## Comparison and discussion

It should be pointed out that, as can be seen in Fig. 1, the signal sequences of the genus *Bacillus* are long (29 to 41 amino acid residues), while those of the enzyme of higher animals are relatively short (12 to 15 amino acid residues); the signal sequence of amylase from barley has, however, not been determined (Rogers and Milliman 1983).

Homology of amino acid residues between two α-amylases is defined as the ratio of the number of identical amino acid residues to the total number of residues of the smaller sequence. The homology was 80—90% in 6 different animal α-amylases. Homology among the α-amylases from a wider range of living organisms — microorganisms, plants and animals, was no more than 10%; an exception was the 60% homology between *B. stearothermophilus* and *B. amyloliquefaciens* α-amylases.

It seemed necessary to resort to the use of a computer to search for homologous regions among the different α-amylases. A dot matrix plot for *B. amyloliquefaciens* and *B. stearothermophilus* α-amylases is shown in Fig. 2A, that for α-amylases of *B. subtilis* and *B. stearothermophilus* is shown in Fig. 2B. A clear, diagonal dotted line in Fig. 2A demonstrates a high degree of homology between *B. amyloliquefaciens and B. stearothermophilus* (thermostable) α-amylases. There is, however, no such distinct diagonal line of dotted points in Fig. 2B; close examination, based on the evidence in Fig. 1, showed that regions 2 and 4 could be recognized, although not very clearly. This reflects the low degree of homology between *B. subtilis* (thermolabile) and *B. stearothermophilus* (thermostable) α-amylases.

Since these two regions were found to be shared by all the amylases examined, as is seen in Fig. 1, it was concluded that regions 2 and 4 might function as active and/or substrate-binding sites.

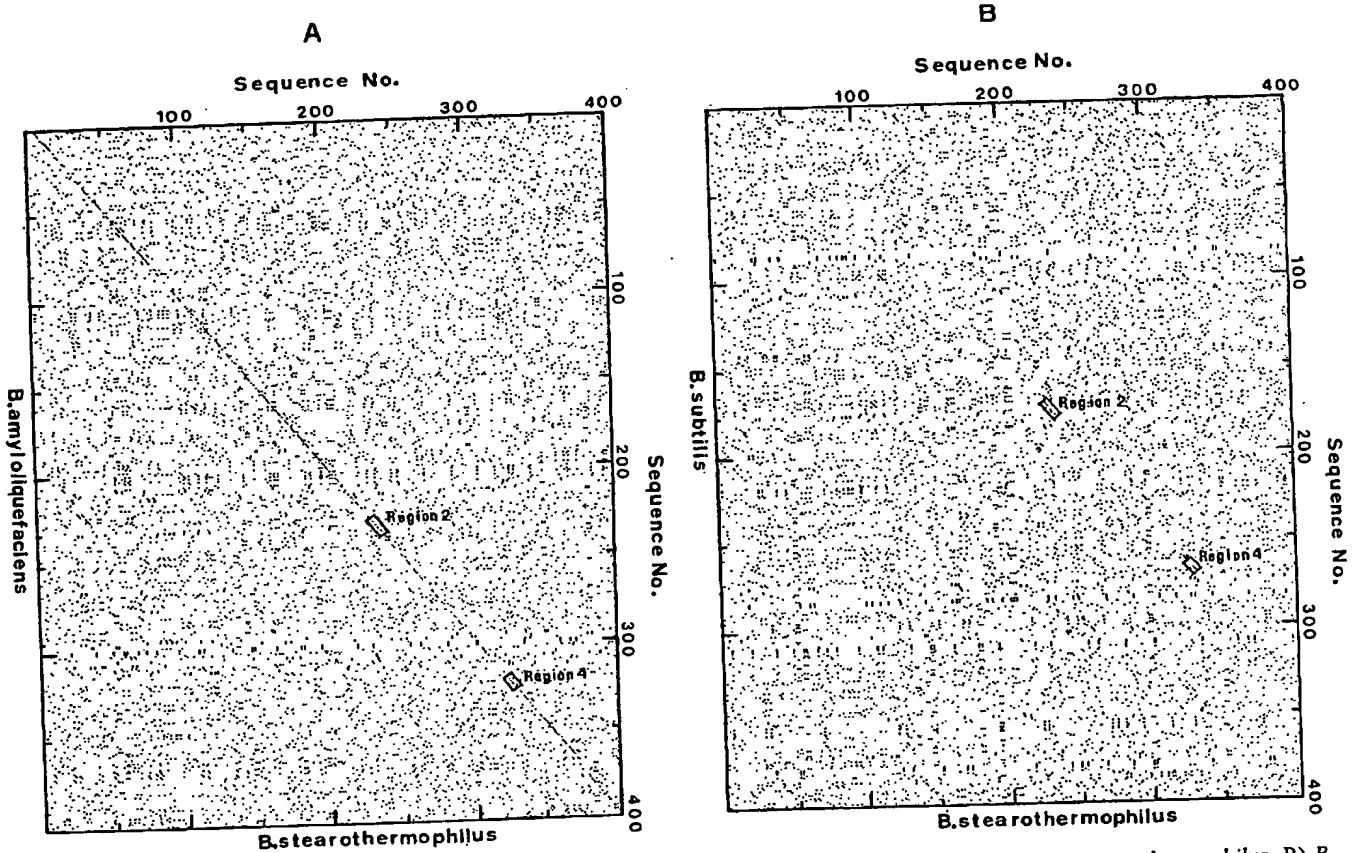



Fig. 2. Computer search for homologous area of the amino acid sequence. A) *B. amyloliquefaciens* vs *B. stearothermophilus*, B) *B. subtilis* vs *B. stearothermophilus*. Areas surrounded by boxes correspond to regions 2 and 4 in Fig. 1. The sequence numbers in both diagrams are for the secreted proteins, counting the 1st amino acid in the 2nd row in Fig. 1 as +1. Regions 1 and 3 are not shown in these diagrams: for these, see text

In fact, a molecular model of Taka-amylase A (α-amylase) from *A. oryzae* by Matsuura et al. (1984) suggests that both His and Asp in these homologous areas function as active sites, while Asp and Lys in region 2 and His in region 4 may function as substrate-binding sites.

Since another active site of Glu and the neighboring substrate-binding sites of Val, Leu and Asp between regions 2 and 4 have been suggested (Matsuura et al. 1984), the sequences were examined for an alignment containing these four amino acid residues. Just such an area has been designated as region 3 in Fig. 1. It is interesting to note that the three active-site amino-acids, His, Glu and Asp, occur consistently in regions 2, 3 and 4, respectively for all the enzymes tested. Many further substrate-binding sites are proposed for Taka-amylase A (Matsuura et al. 1984); these are indicated by the square symbols scattered throughout the entire sequence of amylase from *A. oryzae*.

In the process of locating homologous areas in the amino acid sequences by the trial and error procedure, we found, in addition to regions 2 and 4, another homologous area, designated as region 1 in Fig. 1. The fact that only these four homologous regions 1, 2, 3 and 4 were found consistently, despite the different origins of the organisms, whether bacteria, fungi, higher plants and animals, suggests that these regions are indispensable for the expression of α-amylase activity.

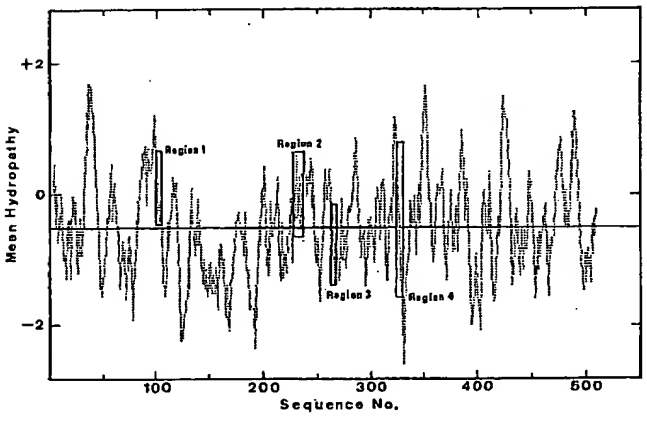



**Fig. 3.** Hydropathy profile of *B. stearothermophilus* α-amylase. The mean hydropathy (Kyte and Doolittle 1982) of a moving segment of nine amino acid residues is shown. The sequence No. is defined as before. The line parallel to the abscissa represents the average of hydropathy of this enzyme. The areas corresponding to regions 1, 2, 3 and 4 in Fig. 1 are surrounded by boxes

Figure 3 shows the hydropathic character of α-amylase from *B. stearothermophilus*. Hydrophobicity and hydrophilicity of the enzyme are determined by a moving segment of nine amino acid residues along the sequence. The parts that project above the solid line in the figure denote strongly hydrophobic and interior regions of the enzyme, whereas those that project below the line designate strongly hydrophilic segments and the exterior. Regions 1, 2, 3 and 4 reproduced in Fig. 3 are located in the valley of protein surface between the exterior and interior regions. This supports the suggestion that these regions are important for the catalytic activity of α-amylase.

Another characteristic difference between the thermostable enzymes of *B. stearothermophilus*, *B. amyloliquefaciens* and the thermolabile enzyme from *B. subtilis* is the distance between regions 1 and 2 (Fig. 1). These differences may indicate a possible line of research on the complex nature of thermophily.

## References

Hagenbüchle O, Bovey R, Young RA (1980) Tissue-specific expression of mouse α-amylase genes: nucleotide sequence of isozyme mRNAs from pancreas and salivary gland. Cell 21:179—187

Kluh I (1981) Amino acid sequence of hog pancreatic α-amylase isoenzyme I. FEBS Lett 136:231—234

Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157:105—132

MacDonald RJ, Crerar MM, Swain WF, Pictet RL, Thomas G, Rutter WJ (1980) Structure of a family of rat amylase genes. Nature 287:117—122

MacDonald RJ, Stary SJ, Swift GH (1982) Two similar but nonallelic rat pancreatic trypsinogens: nucleotide sequences of the cloned cDNAs. J Biol Chem 257:9724—9732

Matsumura M, Katakura Y, Imanaka T, Aiba S (1984) Enzymatic and nucleotide sequence studies of a kanamycin-inactivating enzyme encoded by a plasmid from thermophilic bacilli in comparison with that encoded by plasmid pUB110. J Bacteriol 160:413—420

Matsuura Y, Kusunoki M, Harada W, Kakudo M (1984) Structure and possible catalytic residues of Taka-amylase A. J Biochem 95:697—702

Nakajima R, Imanaka T, Aiba S (1985) Nucleotide sequence of the *Bacillus stearothermophilus* α-amylase gene. J Bacteriol 163:401—406

Nakamura Y, Ogawa M. Nishide T, Emi M, Kosaki G, Himeno S, Matsubara K (1984) Sequences of cDNAs for human salivary and pancreatic α-amylases. Gene 28:263—270

Novotny J (1982) Matrix program to analyze primary structure homology. Nucleic Acids Res 10:127—131

Rogers JC, Milliman C (1983) Isolation and sequence analysis of a barley α-amylase cDNA clone. J Biol Chem 258:8169—8174

Takkinen K, Pettersson RF, Kalkkinen N, Palva I, Söderlund H, Kääriäinen L (1983) Amino acid sequence of α-amylase from *Bacillus amyloliquefaciens* deduced from the nucleotide sequence of the cloned gene. J Biol Chem 258:1007—1013

Toda H, Kondo K, Narita K (1982) The complete amino acid sequence of Taka-amylase A. Proc Japan Acad 58:208—212

Yamazaki H, Ohmura K, Nakayama A, Takeichi Y, Otozai K, Yamasaki M, Tamura G, Yamane K (1983) α-Amylase genes (*amyR2* and *amyE*$^+$) from an α-amylase-hyperproducing *Bacillus subtilis* strain: Molecular cloning and nucleotide sequences. J Bacteriol 156:327—337

Yang M, Galizzi A. Henner D (1983) Nucleotide sequence of the amylase gene from *Bacillus subtilis*. Nucleic Acids Res 11:237—249

Yutani K, Ogasahara K, Sugino Y, Matsushiro A (1977) Effect of a single amino acid substitution on stability of conformation of a protein. Nature 267:274—275

Received July 25, 1985/Revised September 12, 1985